



Nanoparticle-based bio-barcode assay redefines "undetectable" PSA and biochemical recurrence after radical prostatectomy

C. Shad Thaxton^{a,b,1}, Robert Elghanian^c, Audrey D. Thomas^a, Savka I. Stoeva^c, Jae-Seung Lee^c, Norm D. Smith^{a,b}, Anthony J. Schaeffer^{a,b}, Helmut Klocker^d, Wolfgang Horninger^d, Georg Bartsch^d, and Chad A. Mirkin^{b,c,1}

^aDepartment of Urology, Northwestern University Feinberg School of Medicine, 303 East Chicago, Chicago, IL 60611; ^bRobert H. Lurie Comprehensive Cancer Center, Northwestern University, Galter Pavilion, 675 North Saint Clair, 21st Floor, Chicago, IL 60611; ^cDepartment of Chemistry, International Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208; and ^dDepartment of Urology, Innsbruck Medical University, Innsbruck, Tyrol, Austria

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We report the development of a previously undescribed gold nanoparticle bio-barcode assay probe for the detection of prostate specific antigen (PSA) at 330 fg/mL, automation of the assay, and the results of a clinical pilot study designed to assess the ability of the assay to detect PSA in the serum of 18 men who have undergone radical prostatectomy for prostate cancer. Due to a lack of sensitivity, available PSA immunoassays are often not capable of detecting PSA in the serum of men after radical prostatectomy. This new bio-barcode PSA assay is ≈ 300 times more sensitive than commercial immunoassays. Significantly, with the barcode assay, every patient in this cohort had a measurable serum PSA level after radical prostatectomy. Patients were separated into categories based on PSA levels as a function of time. One group of patients showed low levels of PSA with no significant increase with time and did not recur. Others showed, at some point postprostatectomy, rising PSA levels. The majority recurred. Therefore, this new ultrasensitive assay points to significant possible outcomes: (i) The ability to tell patients, who have undetectable PSA levels with conventional assays, but detectable and nonrising levels with the barcode assay, that their cancer will not recur. (ii) The ability to assign recurrence earlier because of the ability to measure increasing levels of PSA before conventional tools can make such assignments. (iii) The ability to use PSA levels that are not detectable with conventional assays to follow the response of patients to adjuvant or salvage therapies.

carcinoma of prostate | prostate specific antigen

Carcinoma of the prostate (CaP) is the most common noncutaneous malignancy among American men and is the second leading cause of cancer death in the United States (1). Prostate specific antigen (PSA) is a serum biomarker used for CaP screening, and is near exclusively a product of the physiology and pathophysiology of prostatic epithelial cells (2). Commercially available PSA immunoassays, with clinical lower limits of detection down to 0.1 ng PSA/mL serum, accurately quantify PSA serum levels within the range needed for screening purposes (3).

Although some researchers and clinicians argue about the merits of using PSA levels as a routine screening tool for prostate cancer (4, 5), PSA can be used as an unambiguous indicator of response to therapy and recurrence in the case of patients who have undergone radical prostatectomy (6, 7). Biochemical recurrence after CaP treatment is defined as a PSA level rising from <0.1 ng/mL to persistently >0.2 ng/mL, and occurs in up to 40% of men who are surgically treated (3, 7–9). For pathologically aggressive and recurrent CaP, a number of studies have concluded that early adjuvant or salvage radiation treatment delivered after radical prostatectomy leads to significant improvements in patient outcomes (10, 11). Also, intervention at low PSA values was significantly associated with improved

outcomes in these studies (10, 11). In contrast to CaP screening where serum PSA values are within a measurable range, radical prostatectomy eliminates the source of PSA production from the standpoint of commercially available PSA immunoassays, and serum values characteristically fall below the 0.1 ng/mL limit of detection. The same is often the case for patients receiving adjuvant or salvage therapy for CaP. As a result, clinicians and patients are not able to prospectively assess whether one is disease free or is destined for recurrence, nor can there be an objective assessment of the biochemical response to adjuvant or salvage treatments. In light of the clinical data, the ability to reliably and accurately quantify PSA values <0.1 ng/mL may enable a more timely assessment of the response to primary therapy, promptly direct the delivery of beneficial adjuvant or salvage treatments, and allow researchers to validate new therapies and assess the biochemical response to such interventions.

Ultrasensitive prototype assays have been used to retrospectively interrogate the serum of men postprostatectomy to test the hypothesis that more sensitive PSA assays increase the prevalence of postprostatectomy PSA detection and identify biochemical recurrence with substantial lead times (12–14). Despite this data and the continued clinical need to rapidly identify disease recurrence, such an assay has not been commercialized and used for further studies.

The bio-barcode assay (Scheme 1) is an emerging diagnostic tool, based on advances in nanotechnology, used for the enzyme-free ultrasensitive detection of various protein and nucleic acid targets (15–18). In the case of proteins, the bio-barcode assay can be between one and six orders of magnitude more sensitive than conventional ELISA-based assays, depending on target and sample complexity (15–18). This increase in sensitivity offers the opportunity to monitor existing biomarkers at levels not possible with conventional assays. The utility of the bio-barcode assay for detecting bio-molecules at extremely low concentrations was originally demonstrated with PSA (15). Advances were needed to assess the benefits of the bio-barcode assay in a clinical setting. Accordingly, we report the design and synthesis of a previously undescribed and more robust nanoparticle probe, the automation of the bio-barcode assay to support the interrogation of

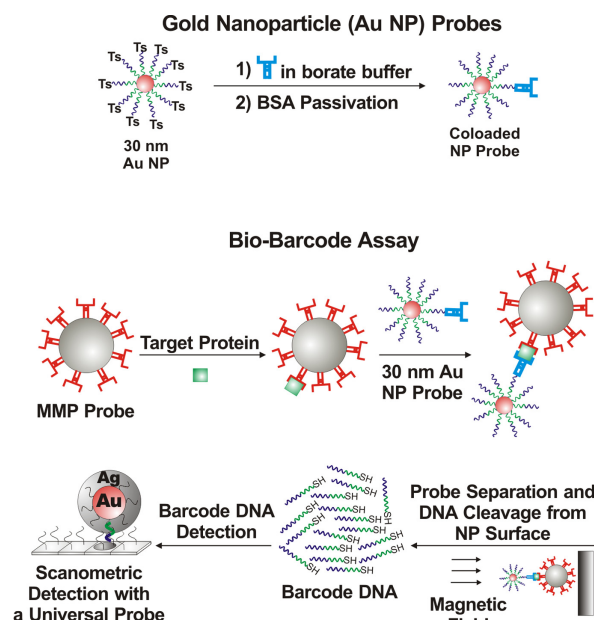
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¹To whom correspondence may be addressed. E-mail: cthaxton003@md.northwestern.edu or chadnano@northwestern.edu.

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Scheme 1. Schematic representation of the PSA Au-NP probes (Upper) and the PSA bio-barcode assay (Lower). (Upper) Barcode DNA-functionalized Au-NPs (30 nm) are conjugated to PSA-specific antibodies through barcode terminal thiol (Ts) modification to generate the coloaded PSA Au-NP probes. In a second step, the PSA Au-NP probes are passivated with BSA. (Lower) The bio-barcode assay is a sandwich immunoassay. First, MMPs surface-functionalized with monoclonal antibodies to PSA are mixed with the PSA target protein. The MMP-PSA hybrid structures are washed free of excess serum components and resuspended in buffer. Next PSA Au-NP probes are added to sandwich the MMP-bound PSA. Again after magnetic separation and wash steps, the PSA-specific DNA barcodes are released into solution and detected using the scanometric assay, which takes advantage of Au-NP catalyzed silver enhancement. Approximately $\frac{1}{2}$ of the barcode DNA sequence (green) is complementary to the “universal” scanometric Au-NP probe DNA, and the other $\frac{1}{2}$ (purple) is complementary to a chip-surface immobilized DNA sequence that is responsible for sorting and binding barcodes complementary to the PSA barcode sequence.

many serum samples in replicate, and PSA detection in human serum matrices. The lower limit of detection for PSA in this format is ≈ 300 times more sensitive than the 0.1 ng/mL clinical limit of detection for commercial assays (3).

In addition to assay improvements, we also sought to determine whether the bio-barcode assay would provide information beyond that of current commercial PSA assays in the context of clinical PSA detection. Here, we report an example of the bio-barcode assay performed in human serum, and the results of a pilot study assessing PSA levels in the serum of men who have undergone radical prostatectomy (RRP) for clinically localized prostate cancer (Table 1) (19). Using the bio-barcode assay, we demonstrate the following. (i) There is a detectable non-zero PSA level in all men in this patient cohort after radical prostatectomy. (ii) Some men are found to have no evidence of CaP, because they show low and nonrising levels of PSA. (iii) PSA recurrence can be detected earlier using the bio-barcode PSA assay. (iv) The bio-barcode assay is capable of assessing the biochemical response to salvage therapy.

Results

Bio-Barcode PSA Gold Nanoparticle (Au-NP) Probe. In this novel Au-NP PSA probe design, PSA-specific antibodies are covalently attached to the surface of Au-NPs through terminal barcode

DNA thiol modification (Scheme 1). A quality control test for the PSA Au-NP probe was used to confirm particle functionality and stability before testing patient serum (*SI Materials and Methods*) (Fig. S1).

PSA Assay Serum Matrix and Assay Calibration. The bio-barcode PSA assay and assay calibration were developed in human female serum (*SI Materials and Methods*) (Fig. S2). Calibration curves spanned the PSA concentration range between 330 fg/mL and 33 pg/mL by spiking known amounts of WHO 90:10 PSA into female serum and carrying out the PSA bio-barcode assay. Fig. 1 demonstrates an example PSA calibration curve and the gray scale Scanometric assay response to the calibrator series and to the negative control human female serum (i.e., no PSA added).

Patient Data. All patient sample data obtained using the bio-barcode assay are provided and compared with values generated using the commercial assays (Figs. 2–4). The commercial immunoassays used to determine the postprostatectomy PSA serum values in the patient cohort changed during the period of patient follow-up. The Abbott IMx assay was used until 2001 and the Bayer Centaur assay was used thereafter. In each case, the clinical limit of PSA detection was 0.1 ng/mL. PSA was detectable in the serum of men after radical retropubic prostatectomy in 86% (102/118) of the measured serum samples using the bio-barcode assay (Figs. 2–4), as compared with 25% (30/118) using the commercial assays (see above).

In nonrecurrent patients 1–9, the commercial PSA assays (see above) used to screen the patient samples reported undetectable PSA values in every case (Fig. 2). Patients 1–7 in the nonrecurrent group had low and nonrising postoperative PSA values when measured with the bio-barcode assay throughout the serial sampling period (Fig. 2). Patients 8 and 9 demonstrate an increase in their postoperative PSA values in the <100 pg/mL range as measured with the bio-barcode assay (Fig. 2), whereas remaining in the undetectable range as measured with the commercial assays (see above).

The bio-barcode assay provided a lead time in the detection of a rising PSA level and eventual biochemical recurrence in patients 10 and 11 (Fig. 3).

There were three patients with initially undetectable PSA values, who then experience rapid biochemical recurrence (patients 12–14; Fig. 3). Patient 15 (Fig. 3) provides an example in whom it appears that the patient-specific PSA nadir is close to 100 pg/mL for ≈ 2 years postoperatively. The bio-barcode assay tracks this patient's PSA values, whereas the ELISA reports alternating undetectable and detectable values before demonstrating a clear trend toward biochemical recurrence (Fig. 3).

Patients 16–18 in the recurrent patient group had documented disease persistence and PSA values that were detectable immediately after surgery (Fig. 4). For patients 16 and 18, serum samples before the delivery of adjuvant treatment were not provided for analysis. The bio-barcode assay tracks the biochemical response to salvage intervention (Fig. 4).

Discussion

PSA Au-NP Probes. Previous to this work, PSA Au-NP probes were fabricated by coadsorbing thiol-terminated DNA barcodes and anti-PSA antibodies to the surface of Au-NPs (15, 17). This approach intuitively limits the Au-NP loading of barcode DNA due to the occupation of the Au-NP surface by adsorbed antibodies, and limits probe stability due to the reduced shelf-life of antibodies as compared with DNA. In the synthetic approach reported here, the loading of oligonucleotide barcodes onto the Au-NP surface before antibody loading provides maximum surface area for loading the signal-generating barcode strands to the surface of Au-NP probes, provides a stable NP platform for antibody addition that is amenable to long-term storage, and

Table 1. Patient data

Patient	Follow-up, years	Pre-RRP PSA, ng/mL	Pathologic Gleason score	Pathologic stage	Postoperative treatment
1	7.2	7.1	3 + 4 = 7	T2b	No
2	9.0	3.8	3 + 3 = 6	T2a	No
3	6.3	5.8	2 + 3 = 5	T2b	No
4	5.2	2.7	3 + 3 = 6	T2b	No
5	4.8	5.9	2 + 3 = 5	T2a	ND
6	9.2	0.7	2 + 4 = 6	T2c	ND
7	6.2	7.7	2 + 3 = 5	T2a	ND
8	8.0	13.1	4 + 4 = 8	T3a	No
9	5.7	1.0	3 + 4 = 7	T2b	ND
Median	6.3				
10	6.2	5.0	3 + 3 = 6	T2a	No
11	5.4	3.7	3 + 3 = 6	T2b	Yes
12	9.1	4.8	3 + 3 = 6	T2b	No
13	8.5	8.0	3 + 4 = 7	T3b	No
14	5.0	4.3	3 + 3 = 6	T3a	Yes
15	5.6	11.1	3 + 4 = 7	T2b	Yes
16	5.1	12.8	4 + 4 = 8	T3a	Yes
17	5.3	3.1	3 + 3 = 6	T2a	ND
18	5.4	6.5	3 + 4 = 7	T3a	Yes
Median	5.4				

Clinical data. ND, not determined.

provides consistent barcode loading to Au-NPs. This new nanoparticle conjugate design and the tosyl functionalization (20, 21) enables nucleophilic substitution and covalent antibody attachment to the Au-NP surface indirectly through the immobilized barcode DNA sequences. The dithiol moiety on the terminus opposite the tosyl group allows for selective binding of the oligonucleotide to the Au-NP surface and enhances particle

stability through disulfide conjugation (22, 23). Tosyl functional groups are sensitive to monothiol coupling; thus, a disulfide was a necessary modifier in the presence of the tosyl leaving group.

Assay Development for Human Serum Samples. The use of human female serum in our calibration curves enabled us to optimize the bio-barcode assay in a “blank” matrix similar to that of the unknown patient samples but lacking detectable PSA, and also ensured the stability of all assay components before running patient serum samples. Human female serum can contain PSA when interrogated with an ultrasensitive PSA immunoassay (24). The lots of female sera used for this study were found to have undetectable PSA-specific signal at a 30% dilution (*SI Materials and Methods*). In addition to PSA-specific background signal, human serum may contain antibodies that generate spurious immunoassay results (25, 26). Also, nonspecific binding events between PSA Au-NP probes and magnetic particles can be a source of nonspecific background signal. We used a commercially available blocking agent to inhibit nonspecific assay signal generated by cross-reacting human antibodies, and added polyacrylic acid (PAA) to the assay buffers to diminish background signal caused by PSA Au-NP probes binding nonspecifically to magnetic particles (*SI Materials and Methods*).

Patient Data. Biochemical CaP recurrence is common after radical prostatectomy, and is often followed by clinical recurrence, metastatic disease, morbidity, and death from CaP (8, 27). Accordingly, a significant proportion of men who have undergone therapy for clinically localized disease go on to receive some form of adjuvant or salvage treatment (27–29). Recent studies indicate that early adjuvant and salvage radiation treatments significantly improve prostate cancer specific survival and overall survival (10, 11). Despite the apparent benefits of early detection and treatment, and data demonstrating that PSA is present in the serum after CaP treatment at values <0.1 ng/mL (12–14), there has never been a clinical trial where treatment was delivered based on PSA values <0.1 ng/mL. Utilization of commercial assays with reported limits of detection <0.1 ng/mL to screen the serum of men after prostatectomy has led to the conclusion that assay noise in the 1–100 pg/mL region precludes

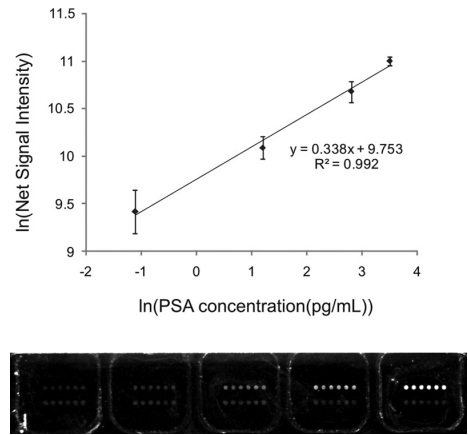


Fig. 1. Representative PSA calibration curve. The calibrator samples were prepared by spiking 0.0, 0.1, 1.0, 5.0, and 10.0 pg/mL of WHO 90:10 PSA standard into human female serum prescreened for PSA (*SI Materials and Methods*) (Fig. S2). Assuming 100% serum, the final PSA concentrations correspond to a 0.33, 3.3, 16.6, and 33.3 pg/mL calibrator series, accordingly. Samples were run on the automated system with subsequent scanometric detection of the barcode DNA strands released from the 30 nm Au NP probes for PSA target detection and quantification. An example of the gray scale scanometric readout results is shown. The top row of spots in each 2 × 6 well set is the assay response to PSA barcode DNA obtained from the immunoassay, and the bottom set of six spots is the scanometric assay calibrator response. The zero calibrator (no PSA added) is on the far left, with increasing PSA concentrations, as above, moving to the right. The scanometric DNA calibrator sequence is added at the same concentration in each well set.

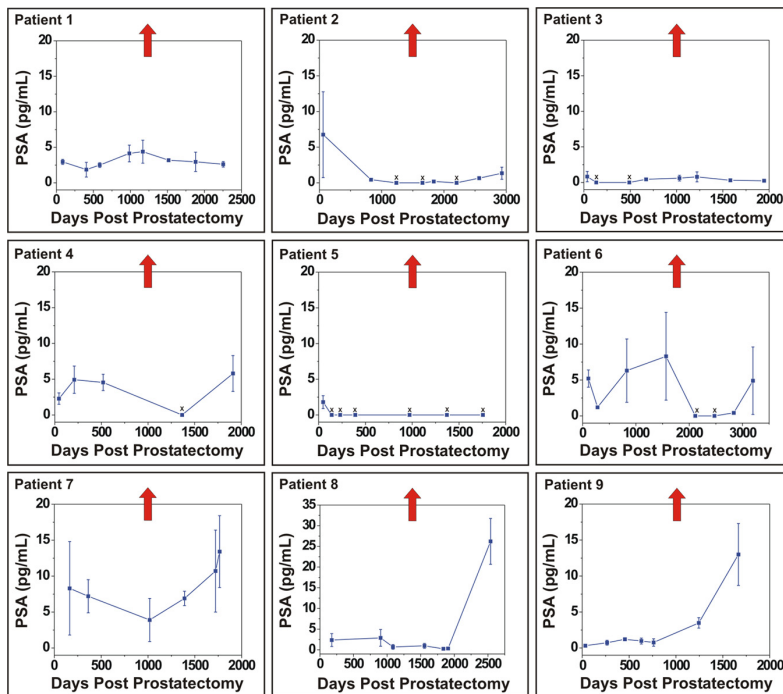


Fig. 2. Nonrecurrent patients 1–9. Postprostatectomy PSA concentrations (pg/mL) as measured with the bio-barcode assay (blue squares). ELISA limit of detection (0.1 ng/mL) is outside the scale of the graph as indicated by the red arrow. Xs indicate samples that are undetectable with the bio-barcode assay.

the attainment of clinically meaningful results (3, 30). However, having an undetectable postprostatectomy serum value using commercial ultrasensitive assays portends a good prognosis (31, 32). Here, we demonstrate that the bio-barcode assay not only allows one to assign a patient-specific PSA nadir value <0.1 ng/mL, but also allows one to quantitatively track PSA concen-

trations in serum from 330 fg/mL to higher values. Through this enabling technology, a thorough investigation of the clinical utility of this capability is possible.

The data described here demonstrate that the PSA bio-barcode assay allows for the diagnosis of either a no evidence of disease status or disease relapse at the earliest possible time in

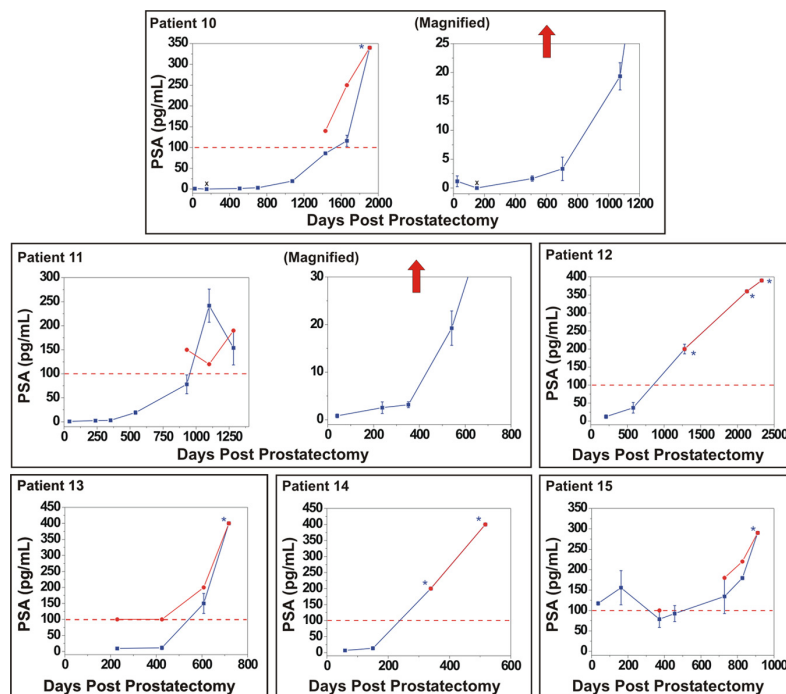


Fig. 3. Recurrent patients 10–15, who had undetectable PSA values on the first postoperative sample when measured with conventional assays (the commercial immunoassays used to determine the postprostatectomy PSA serum values in the patient cohort changed during the period of patient follow-up. The Abbott IMx assay was used until 2001 and the Bayer Centaur assay was used thereafter. In each case, the clinical limit of PSA detection was 0.1 ng/mL). Postprostatectomy PSA concentrations (pg/mL) as measured with the bio-barcode assay (blue squares) and the commercial assays (red dots) (see above). The clinical limit of detection (0.1 ng/mL) is indicated by a red dotted line, or red arrow when outside the scale of the graph. Xs indicate samples that are undetectable with the bio-barcode assay. Blue asterisks indicate that the PSA value was above the optimal detection range for the bio-barcode assay; therefore, the ELISA value was used.

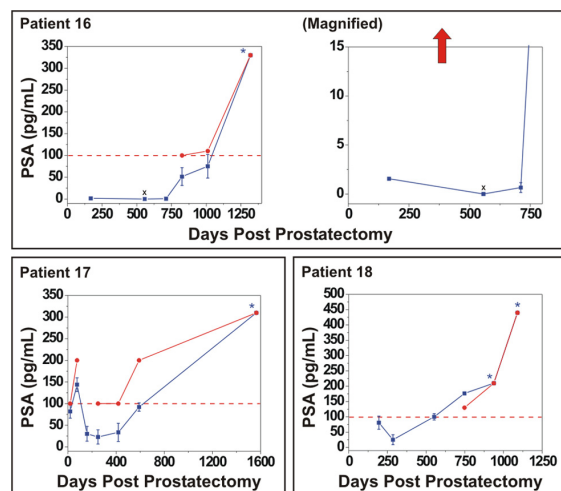


Fig. 4. Recurrent patients 16–18, who demonstrated persistent PSA elevations after surgery. Postprostatectomy PSA concentrations (pg/mL) measured with the bio-barcode assay (blue squares) and the commercial assays (red dots). Clinical limit of detection (0.1 ng/mL) is indicated by a red dotted line, or red arrow when outside the scale of the graph. Xs indicate samples that are undetectable with the bio-barcode assay. Blue asterisks indicate that the PSA value was above the optimal detection range for the bio-barcode assay; therefore, the ELISA value was used.

the patient cohort studied. The assay measured PSA in the serum of every individual in this patient cohort after radical prostatectomy, and in 87% of the samples. According to our data, a PSA level <5 pg/mL may represent the nadir at or under which men with no evidence of residual disease reside, equivalent to a “normal” PSA in a man after radical prostatectomy. In these patients, the bio-barcode assay could potentially be used to reassure an individual with regard to a no evidence of disease status. Such patients may be excellent candidates for altered postoperative surveillance protocols. The source of low-level and nonrising PSA in the serum of these patients is uncertain (2, 33). Interrogating serum samples from a larger cohort of patients would more clearly define what PSA level is physiologic in the prostatectomized patient.

In our cohort of patients, there is clearly a number of men in whom ultrasensitive PSA monitoring using the bio-barcode assay allows for the detection of a rise in the PSA level earlier than commercially available assays. In the nonrecurrent group, patients 8 and 9 demonstrate late PSA increases in the PSA range <0.1 ng/mL, whereas at the same time, are undetectable with conventional assays (Fig. 2). Also, patients 10 and 11 demonstrate a clear bio-barcode trend of increasing PSA in the ultrasensitive range, and eventually, experience biochemical recurrence as measured with conventional assays (Fig. 3). In the former case, the bio-barcode data suggests that these patients will recur; however, further data would be necessary to make definitive clinical conclusions regarding disease recurrence. In the latter cases, the bio-barcode provides a lead time in the diagnosis of recurrence. In each case, these data strongly argue the need for a prospective study directly comparing ultrasensitive PSA monitoring with commercially available systems to adequately address the clinical benefit of this capability.

Patients 12–14 have PSA values that were initially undetectable, but demonstrate rapid postoperative PSA recurrence. In these cases, the bio-barcode assay tracks the PSA profiles, but is incapable of providing a recurrence lead time that is superior to the commercial assays. Our data allow us to hypothesize that if the sampling times in these patients had been more frequent,

especially in the year after treatment, a PSA profile consistent with disease recurrence would have been revealed earlier. Because patients with rapid PSA recurrence and postoperative PSA doubling times are those most likely to die of their disease (7), and are those who may derive the most benefit from early intervention (11), our study argues for a prospective evaluation of all patients after treatment so that the time interval of serum sampling can be controlled postoperatively for the rapid identification of those patients who may benefit most from early detection.

Unfortunately, commercially available assays do not allow one to assess a patient’s response to adjuvant and/or salvage treatment when therapy drives PSA values <0.1 ng/mL. There are three patients in the recurrent cohort with disease persistence after prostatectomy (Fig. 4). In two cases (patients 16 and 18), patients underwent salvage treatment and their PSA values became undetectable. The bio-barcode assay quantitatively tracks the response of these patients to salvage intervention, providing an ultrasensitive biochemical signature of response.

In conclusion, we have synthesized and characterized a new PSA Au-NP bio-barcode assay probe and demonstrated that it can be used in a novel automated format. This assay is ≈ 300 times more sensitive than analogous conventional methods. This increase in sensitivity may provide physicians an opportunity to more closely monitor PSA serum values in patients who have undergone treatment for prostate cancer, and permit researchers the ability to validate new therapies for the disease. With an assay sensitivity of 330 fg/mL, the bio-barcode assay increases the number of patients, post prostatectomy, with measurable PSA in their serum. Our data demonstrate that patients who have postoperative and persistent PSA values <5 pg/mL may be reassured of their no evidence of disease status. Patients who have steadily rising PSA values as measured with the bio-barcode assay are likely en route to biochemical CaP recurrence. Accordingly, our data argue for the more frequent monitoring of postoperative PSA values with the bio-barcode assay, or one of comparable sensitivity, so that recurrence can be detected at the earliest possible time. Frequent monitoring may be especially important in patients with high risk disease who are often subjected to adjuvant and salvage therapies. Nanotechnology-enabled ultrasensitive biomolecular detection offers new opportunities for innovative treatment modalities to be studied in clinical trials with an ultrasensitive measure of disease recurrence and disease response to adjuvant or salvage treatments, and for altering surveillance protocols in those individuals with PSA profiles consistent with disease cure.

Methods

Synthesis of the Bifunctional Oligonucleotide Barcode. The bifunctional oligonucleotide barcode DNA (Table S1) was synthesized on a 1- μ mol scale using standard phosphoramidite coupling on an automated DNA synthesizer (Expedite) using ultramild reagents. All reagents required for DNA synthesis were purchased from Glen Research. For details regarding barcode DNA synthesis and purification, see *SI Materials and Methods*.

PSA Au-NP Probe Preparation. Functionalization of 30 nm Au-NPs with bifunctional oligonucleotide barcodes. Gold nanoparticles with an average diameter of 30 nm were purchased from Ted Pella and used as received at a concentration of ≈ 330 pM ($\approx 2 \times 10^{11}$ particles/mL). The 30 nm Au-NPs were functionalized with the bifunctional oligonucleotides by adding the oligonucleotide to the Au colloid at ≈ 3 μ M final concentration (1 OD DNA per 1 mL of Au colloid). The colloid incubated for 24 h. Next, 10 wt % SDS (Sigma) was introduced at a final concentration of 0.1%, and the NaCl concentration was brought to 0.1 M in one step by adding the appropriate volume of 1M NaCl while vortexing the mixture. After 48 h of incubation, the barcode-functionalized Au-NPs were isolated by centrifugation (6,800 rpm, 15 min) using an Eppendorf bench top centrifuge, washed twice with nuclease-free water (Ambion), and resuspended at the original concentration in water, and stored at 4 $^{\circ}$ C.

Antibody conjugation. Before the antibody conjugation step, 3.0 mL of the 30 nm Au NPs functionalized with tosyl-terminated oligonucleotides were con-

centrated to 60 μL using centrifugation. Tween-20 solution (20 μL 0.2 wt % in water) was then added, followed by addition of 10 μg anti-PSA (goat) affinity purified polyclonal antibody (AF1344; R&D systems) in 20 μL of 1 $\times$ Dulbecco's PBS, pH 7.4 (Invitrogen). The solution was brought to 0.1 M borate buffer concentration by adding 100 μL of 0.2 M borate buffer, pH 9.5. The conjugation was carried out in a 1.5 mL Eppendorf tube for 24 h under vortex at 550 rpm at 37 $^{\circ}\text{C}$. Next, the Au-NPs were passivated by the addition of 10 μL of 10 wt % BSA (R&D systems) and incubated under vortex at 550 rpm for 24 h. The 30 nm Au-NP probes were isolated by centrifugation and washed twice with 1 $\times$ Dulbecco's PBS buffer containing 0.1% BSA/0.025% Tween 20 (assay buffer). The Au-NP probes were finally resuspended in 3 mL of the assay buffer and stored at 4 $^{\circ}\text{C}$. The Au-NP probe concentration was determined by measuring the absorbance of the colloid at 520 nm (34).

Preparation of Magnetic Microparticle Probes (MMPs). Tosyl-functionalized MMPs (1- μm diameter; Invitrogen) were functionalized with monoclonal anti-PSA antibodies (ab403; Abcam) at 37 $^{\circ}\text{C}$ in borate buffer solution, pH 9.5, passivated with BSA, and stored in assay buffer at 10 mg/mL at 4 $^{\circ}\text{C}$. The detailed procedure can be found in the published literature (34).

Serum Samples. We obtained banked post radical prostatectomy serum samples collected prospectively from 18 men who had undergone surgery for clinically localized prostate cancer with curative intent. Patient characteristics, clinical data, and follow-up data are found in Table 1. All of the patients were diagnosed with prostate cancer through a PSA screening study for the early detection of prostate cancer (19). The nonrecurrent men had persistently undetectable serum PSA levels when measured with conventional assays (see above). Nine of the men developed PSA evidence of recurrence (i.e., biochemical recurrence) defined as a postprostatectomy PSA value >0.2 ng/mL. The banked serum samples were provided from the Department of Urology, Innsbruck Medical University (H.K., W.H., and G.B.). The ethics committee at Innsbruck Medical University approved the use of the archival patient serum samples used in this study.

PSA Bio-Barcode Assay. The format of the bio-barcode PSA assay is that of a sandwich immunoassay (Scheme 1). A Perkin-Elmer liquid handling system modified with a magnetic separation device and capable of 96-well throughput was used throughout the testing and patient sample process. The Scano-

metric assay was carried out manually (*SI Materials and Methods*). For a complete description of the PSA bio-barcode assay, see *SI Materials and Methods*.

Scanometric Detection. For details regarding the Scanometric (35–38) detection of barcode and chip calibrator DNA, see *SI Materials and Methods*.

Data Analysis. A master calibration curve was generated using the average net signal intensities per PSA standard run for the entire study. Values for points on the individual standard curves that fell within a range of $\pm 30\%$ of the net signal intensity of the corresponding standard in the master calibration curve were retained. Calibration standards for each run were plotted on a graph of mean net signal intensity versus PSA concentration and fitted with a best-fit trendline from which to calculate unknown patient values. All net scanometric signal intensities were averaged first per well (six spots per well), and then by chip replicate. Patient sample outliers were excluded based on a box plot determination that identified statistical outliers using JMP 5.0 software (SAS Institute). Patient and standard samples were also edited by measuring the signal intensity of scanometric intrawell chip control sequences and barcode net signal intensities to remove clear outliers.

For any patient sample in which the PSA level was determined to be >250 pg/mL by either the bio-barcode assay or the ELISA, the ELISA value was used in place of the bio-barcode value. Restriction to values <250 pg/mL reflects our intent to measure PSA in the target concentration range that is undetectable by commercial assays. Detecting any PSA concentration >250 pg/mL required greater than a 10-fold assay dilution, <3 μL of serum per 100 μL sample, increasing the potential for measurement errors. Thus, we restricted representation of patient data to values <250 pg/mL.

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